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SPERM COMPETITION MECHANISMS, CONFIDENCE OF PATERNITY, AND THE EVOLUTION OF PATERNAL CARE IN THE GOLDEN EGG BUG (*PHYLLOMORPHA LACINIATA*)

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Abstract.—Theoretical models predict how paternal effort should vary depending on confidence of paternity and on the trade-offs between present and future reproduction. In this study we examine patterns of sperm precedence in *Phyllomorpha laciniata* and how confidence of paternity influences the willingness of males to carry eggs. Female golden egg bugs show a flexible pattern of oviposition behavior, which results in some eggs being carried by adults (mainly males) and some being laid on plants, where mortality rates are very high. Adults are more vulnerable to predators when carrying eggs; thus, it has been suggested that males should only accept eggs if there are chances that at least some of the eggs will be their true genetic offspring. We determined the confidence of paternity for naturally occurring individuals and its variation with the time. Paternity of eggs fertilized by the last males to mate with females previously mated in the field has been determined using amplified fragment length polymorphisms (AFLPs). The exclusion probability was 98%, showing that AFLP markers are suitable for paternity assignment. Sperm mixing seems the most likely mechanism of sperm competition, because the last male to copulate with field females sires an average of 43% of the eggs laid during the next five days. More importantly, the proportion of eggs sired does not change significantly during that period. We argue that intermediate levels of paternity can select for paternal care in this system because: (1) benefits of care in terms of offspring survival are very high; (2) males have nothing to gain from decreasing their parental effort in a given reproductive event because sperm mixing makes it difficult for males to reach high paternity levels and males are left with no cues to assess paternity; (3) males cannot chose to care for their offspring exclusively because they can neither discriminate their own eggs, nor can they predict when their own eggs will be produced; and (4) males suffer no loss of further matings with other females when they carry eggs. Thus, our findings do not support the traditional view that paternal investment is expected to arise only in species where confidence of paternity is high. The results suggest that females maximize the chances that several males will accept eggs at different times by promoting a mechanism of sperm mixing that ensures that all males that have copulated with a female have some chance of fathering offspring, that this probability remains constant with time, and that males have no cues as to when their own offspring will be produced.

Key words.—Confidence of paternity, paternal care, paternity, *Phyllomorpha laciniata*, sperm competition, sperm mixing.

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Following Williams (1966) and Trivers (1972), parental care will evolve when the benefits to the fitness of offspring are greater than the costs to the parent's residual reproductive value. Because selection is expected to favor individuals that modulate their investment in relation to genetic paternity, care by males was predicted to arise only in species where confidence of paternity is high (Trivers 1972; Maynard Smith 1977; Gwynne 1984). However, the use of molecular techniques to determine paternity has revealed that, as a result of female polyandry, males often care for unrelated young (e.g., Birkhead and Møller 1992; Møller and Birkhead 1993; Scott and Williams 1993; Westneat and Sargent 1996; Hughes 1998; Sheldon 2002). Such unexpected results prompted a number of theoretical and empirical studies aimed at a better understanding of the relationship between paternity (relatedness to offspring) and parental effort. Whereas some of these studies suggested that decreases of paternity levels should have no effect on paternal behavior (Maynard Smith 1977; Grafen 1980; Davies et al. 1992; Wagner et al. 1996; MacDougall-Shackleton and Robertson 1998; see also Whittingham et al. 1992, 1993; Houston 1995), others have found support for the association between confidence of paternity and paternal investment (Werren et al. 1980; Winkler 1987; Burke et al. 1989; Davies et al. 1992; Whittingham et al. 1992; Xia 1992; Westneat and Sherman 1993; Dixon et al. 1994; Briskie et al. 1998; Sheldon and Ellegren 1998; Neff and Gross 2001; for insects, see Smith 1979; Müller and Eggert 1989; Scott and Williams 1993; Simmons et al. 1993; Wright 1998; Simmons and Achmann 2000; Hunt and Simmons 2002).

Theoretical models have shown that the relationship between paternity and parental care is complex (e.g., Whit-

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tingham et al. 1992; Westneat and Sherman 1993; Houston and McNamara 2002; Webb et al. 2002). Current models predict that paternal care depends on confidence of paternity as well as trade-offs between present and future reproduction (Whittingham et al. 1992; Westneat and Sherman 1993; Mauck et al. 1999). In a model developed by Westneat and Sherman (1993), which reconciles previous modeling attempts, the following four predictions were formulated: (1) when males do not know their own paternity and mean paternity is the same for all matings, a probability of siring offspring below one should have no effect on the optimal level of parental effort; (2) when parents can discriminate their own young, overall parental effort should be reduced, but nepotism should increase; (3) when parental behavior is costly to care-givers, paternity should have more effect than when caring is not costly; and (4) paternity should have less effect when care greatly increases offspring survival than when care is not crucial to offspring survival.

In species in which females mate with more than one male, sperm competition mechanisms will determine how male mating behavior and ejaculate expenditure translates into fertilization success and, therefore, how males can assess confidence of paternity (Smith 1984; Birkhead and Møller 1998; Simmons 2001). It was first predicted that male care should be favored in species with last-male sperm precedence (Gwynne 1984), which is associated with a high confidence of paternity for the last male to copulate.

Phyllomorpha laciniata is a good species to test the predictions of Westneat and Sherman (1993) model because males carry eggs that they have sired as well as unrelated eggs. Patterns of male care seem unusual, because males do not restrict egg acceptance to a short period after copulation and they do not seem to engage in mate guarding. Over 5 years of field study we have not observed any instances of male-male aggression, interference, or attempts to control sexual access to females. Thus, egg carrying by males does not seem to be associated with clear paternity assurance mechanisms. Females mate with multiple males within a reproductive cycle and store the sperm in the spermatheca, thus promoting sperm competition. Females lay one egg at a time continuously over the reproductive season (from March until August), and daily egg production ranges between zero and 10 eggs. Some eggs are laid on conspecifics, whereas others are laid on plants, where they develop unattended (Mineo 1984; Kaitala 1996; Gomendio and Reguera 2001). Eggs carried by adults have higher survival rates than eggs laid on plants, given the lower rates of mortality caused by a parasitoid wasp (Reguera and Gomendio 2002). However, egg carrying conveys costs for adults in terms of increased predation rates (Reguera and Gomendio 1999; Kaitala et al. 2000). Females are unable to oviposit on themselves, thus they are forced to search for conspecifics to lay eggs on them in order to increase their fitness (García-González and Gomendio 2003).

Given the costs of egg carrying for adults, males are expected to accept eggs only if there are chances that at least some of the eggs will be their true genetic offspring. The confidence of paternity levels at which males become willing to accept eggs are likely to depend on the variation of this confidence through the time, the costs of carrying eggs for males, and the consequences of no care for the offspring.

The assignment of paternity is a fundamental piece of information in studying patterns of sperm use and the relationship between paternal care and confidence of paternity. Amplified fragment length polymorphism (AFLP) fingerprinting (Vos et al. 1995) is a very reliable technique based on the selective polymerase chain reaction (PCR) amplification of restriction fragments from a total digest of genomic DNA that generates hundreds of genetic markers (Vos and Kuiper 1997; Mueller and Wolfenbarger 1999). However, the utility of the AFLP technique for paternity analysis has been assessed in very few studies (Krauss and Peakall 1998; Krauss 1999; Questiau et al. 1999; and see Gerber et al. 2000). So far, there are no studies using AFLPs to investigate patterns of sperm use. Here we use for the first time AFLP markers to determine sperm precedence patterns. We have two aims in this study: (1) to understand the mechanisms of sperm use in the golden egg bug to determine how male mating behavior translates into male reproductive success; and (2) to understand the link between sperm precedence patterns observed in natural populations, and the extent of egg carrying by male golden egg bugs.

Materials and Methods

Definitions

In the present study, ''paternity'' means the proportion of juveniles that are genetic offspring of a parent (equivalent to Westneat and Sherman's [1993] ''parentage'' and Wright's [1998] ''actual fatherhood''). ''Confidence of paternity'' is a male's average probability of siring offspring following copulation with a given female (Alexander 1974; Simmons 2001), with no implications about an individual's ability to assess his own paternity. This term was used by Wright (1998) as the ''probability of paternity'' and by Westneat and Sherman (1993) as ''reduced parentage.'' We followed the definition used by Westneat and Sherman (1993) of parental behavior: any action by a parent that increases the survival of young (originally by Clutton-Brock 1991). P_n is the proportion of offspring sired by the last male (the *n*th) to mate, which is calculated in most studies for the second male to mate with a female in a double mating trial, that is, P_2 (Boorman and Parker 1976; Birkhead and Møller 1998).

Samples and Experimental Conditions

The study was designed to determine the number of eggs sired by the last male to mate with the female, by collecting copulating pairs in the field and allowing the female to lay eggs during the five days following the end of the copulation.

Eighteen mating pairs were collected in three close localities of central Spain (mean distance among them: 26.1 km): El Espinar (eight pairs collected on 7 June 2000), Robledo de Chavela (eight pairs collected on 12 June 2000), and Navas del Rey (two pairs collected on 14 June 2000). Each time a mating pair was collected we recorded the position and the stage of development of the eggs (assessed by their color) carried by mating males. Individuals were collected at the peak of the reproductive activity in the population. Moni-

toring of the population from the beginning of the reproductive cycle allowed us to determine this particular stage. Data collected throughout five years and a series of preliminary studies examining sperm contained in the females' spermatheca showed that at this stage of the reproductive cycle 100% of all females in the population have mated at least once. Females of this species are polyandrous, and data from laboratory experiments have shown that females mate multiple times with the same and with different males. Observational data from capture-recapture studies carried out in natural populations supports this fact. Thus, it is very likely that females in this study had mated multiple times in the field before the copulation in which they were captured.

Copulation in this insect lasts on average more than 15 h (23 h on average per Kaitala 1998; 32.5 h on average per Reguera 1999; 17 h on average per García-González 2002) and may last for more than 48 h. To avoid interrupting sperm transfer by the mating male, mating pairs were carefully carried in individual plastic vials to the laboratory. Pairs were placed in small petri dishes (5.5-cm diameter) and kept at constant conditions (25°C, lights on from 0800 to 1700 h). For each mating female the start of the 5-day laying period was defined as the end of the current copulation. We checked for the end of copulations at least four times during the day at 0900, 1300, 1700 and 2100 h. To determine the end of each copulation in those instances in which the exact time was not observed, we used the middle point between two intervals. Most copulations lasted for several hours after the pairs were captured in the field, and there were no cases in which copulation was interrupted by handling. Thus, we have no reason to believe that our experimental procedure could have affected the duration of copulation or the rate of sperm transfer.

Once copulation was finished the male was removed and frozen at −80°C, and the female was allowed to lay eggs freely during the subsequent five days. Our aim was to examine sperm precedence patterns in naturally occurring golden egg bugs by determining paternity of eggs laid by females on the plant without confounding effects by male presence (for instance, repeated matings). However, in some cases, females laid eggs on the mating male before we detected the end of copulation. These eggs could be distinguished from those already carried by males at the time they were captured because recently laid eggs are white, and because the position of eggs carried by males when they were captured was registered, allowing individual identification of each egg. Eggs laid on mating males were processed and used to estimate the paternity of eggs that were laid on males soon after copulation has ended.

Throughout all the experimental period, individuals were provided ad libitum daily with fresh branches of the host plant *Paronychia argentea*. Extreme care was taken to make sure that there were no eggs glued to the plant each time it was added to the animals, including the first time when the mating pairs were collected. This prevented any alien egg previously laid on plants by females in the field to enter the container. Eggs laid by females were collected at 24-h intervals after the end of the copulation, for each particular female, and at the same time that host plant replacement was carried out. All eggs were laid on plants except those laid on males immediately after the end of the copulation. Each egg was labeled with the date of laying, and was placed in an Eppendorf tube and checked daily until hatching. Recently emerged nymphs were frozen as well as adults at −80°C.

Some females initially included in our sample were not analyzed because they did not lay eggs during some of the 5 days considered or because the eggs they laid failed to hatch and could not be used for molecular analyses. Our priority was to include females that laid eggs every day that hatched, so we could trace any changes with time in the paternity of the last male to mate. Thus, we analyzed paternity of offspring from seven families to determine sperm precedence patterns along the five experimental days. In addition, to determine the paternity of eggs carried by males we also included in the analyses the adults and the eggs laid on males from another two families in which females did not lay eggs during some of the 5 days. Finally, the male and female from another copulating pair were included to increase the sample size used to calculate allele frequencies. In total, 109 individuals were analyzed (20 adults, 89 nymphs).

DNA Isolation

Genomic DNA was isolated from the thorax of adults previously cleaned from chitinous extensions ($n = 20$ adults) and from the whole body of the nymphs emerged from eggs laid by females from nine families ($n = 89$), using a CTAB procedure modified from Weising et al. (1995, p. 51), Möller et al. (1992), and Reineke et al. (1998). Isolated DNA was diluted in 20 μl of distilled water.

Amplified Fragment Length Polymorphisms Analysis

A total of 109 individuals were processed. AFLPs were resolved using the AFLP™ Plant Mapping Protocol for Genomes 500–6000 Mb (Perkin-Elmer Applied Biosystems, Foster City, CA), but the reactions were performed with half the volume described in the protocol, with the exception of the quantity of DNA and adapters used. In a series of preliminary tests, we verified that the profile of AFLP fragments obtained was identical to that seen using the total reaction volume. All products were purchased from Perkin-Elmer, except enzymes MseI, EcoRI and T4 DNA ligase, which were obtained from New England Biolabs, Inc. (Beverly, MA). Briefly, AFLP technique involved three stages.

(1) Restriction of the DNA and ligation of adapters.—Enzymatic digestion with MseI and EcoRI, frequent and rare cutter, respectively, and ligation of adapters specific to the restriction digest site were performed simultaneously. The adapters place a known sequence at the ends of the restriction fragments (Vos et al. 1995). Isolated DNA (1.10 μl) was used in a 5.5-μl restriction-ligation reaction at 37°C for 3 h. The reaction was then diluted to 100 μl in TE buffer.

(2) Preselective amplification by PCR.—Preselective primers are complimentary to the adapter sequences with the addition of one nucleotide at the 3′ end to amplify only those restriction fragments with a complimentary nucleotide just interior to the restriction site. Two microliters of the diluted restriction-ligation DNA were mixed with 0.5 μl of preselective primers and 7.5 μl of AFLP Core Mix (Perkin-Elmer). PCR was performed as follows: 2 min at 72°C followed by

25 cycles with the following cycle profile: a 1-sec DNA denaturation step at 94°C, a 30-sec annealing step at 56°C, and 2-min extension step at 72°C. A single step of 60°C for 30 min followed before holding at 4°C. Then half of the reaction was diluted to 100 μl in TE buffer, and the other half was used to verify the successful amplification of target sequences via electrophoresis in a 1.5% agarose gel stained with ethidium bromide.

(3) Selective amplification by PCR.—This step uses primers that match the known adapter sequence, plus three selective nucleotides on the 3′ end of the MseI primer and three selective nucleotides on the 3′ end of the fluorescently labeled EcoRI primers (see below). For each of the two primer pairs the following reaction was performed: 7.5 μl of Core Mix, 0.5 μl of primer MseI, 0.5 μl of primer EcoRI, and 1.5 μl of preselective DNA sample from step 2. A touchdown PCR reaction commenced with one cycle of 94°C for 2 min, 65°C for 30 sec, and 72°C for 2 min. In subsequent cycles, the denaturation time was 1 sec and the annealing temperature was reduced in 1°C steps to 57°C, followed by 23 cycles at 56°C. A single step of 60°C for 30 min followed before holding at 4°C.

Two microliters for Green-labeled and 4 μl for Yellow-labeled selective amplification were added to 15 μl of formamide and 0.5 μl of Genescan-500 ROX-labeled size standard. This mixture was denatured 5 min at 95°C and run on an ABI Prism 310 Genetic Analyzer (Perkin-Elmer). In a previous study, 30 different selective primer combinations were tested, and two selective primer pairs were identified as highly polymorphic, offering clean and reproducible patterns: MseI-CAT and EcoRI-AAG (dye label: JOE-Green), and MseI-CAG and EcoRI-AAC (dye label: NED-Yellow). Thus, in this study, we performed selective PCRs for each of these two primer pairs and the final product was run on the Genetic Analyzer. Digitally converted raw data were saved on a computer, and multilocus profiles were visualized using ABI Genescan software.

Data Analysis

Only unambiguous AFLP markers that were easily scored were used. We considered each fragment as a dominant locus with two states: presence or absence. AFLP profiles were scored for the presence or absence of fragments in the 60–300-bp range. The size in base pairs was given by the comigration of a size standard. Two peaks were considered of the same size if they differed by less than 0.5 bp.

In a previous study, a series of sample replications were conducted to check the repeatability of the method. This repeatability reached 96.6% for the combination of the two primer pairs used (F. García-González, Y. Núñez, F. Ponz, E. R. S. Roldán, M. Gomendio, unpub. data).

We have used an index of correlation in migration (IC) for all loci i and j, with $i \neq j$, to check for correlation between loci (Questiau et al. 1999). For this purpose, we calculated all pairwise comparisons between loci for all individuals (N), using two states for each locus: one for presence of a peak, zero for the absence. The index of correlation in migration is:

$$\mathrm{IC} = \sum_{n=1}^{N} |\mathrm{state}_{i\mathrm{th}\ \mathrm{locus}} - \mathrm{state}_{j\mathrm{th}\ \mathrm{locus}}|/N \qquad (1)$$

A value of one between two fragment positions means that when a peak appears at the ith position, another peak does not appear at the jth position or vice versa. A value of zero means identical appearance or absence in both ith and jth position, which could indicate comigration of the two fragments. No correlation in the migration of bands was detected in the two primer pair profiles. A total of 167,860 pairwise comparisons were calculated for MseI-CAT/EcoRI-AAG and 192,494 for MseI-CAG/EcoRI-AAC. No values of zero or one were detected for the sum of all individuals from one to N within each comparison for loci i and j. The average index for MseI-CAT/EcoRI-AAG was $\mathrm{IC}_{\mathrm{Green}} = 0.49 \pm 0.13$ (mean $\pm$ standard deviation, $n = 1540$) and for MseI-CAG/EcoRI-AAC was $\mathrm{IC}_{\mathrm{Yellow}} = 0.34 \pm 0.12$ ($n = 1766$). This indicates the independence of the loci.

Exclusion probability.—We considered only the adult individuals of our sample for the allele frequency calculations (Questiau et al. 1999). We used the proportion of individuals with no peak for a given locus as the genotypic frequency of the recessive homozygotes (q^2), with q being the estimation of the frequency of the allele absence in the population for that locus. We defined p as the frequency of the allele presence with $p = 1 - q$. We focused only on polymorphic loci with $q^2 > 3/N$, with N being the number of adult individuals, as recommended by Lynch and Milligan (1994), a criterion followed also by Questiau et al. (1999).

Exclusion probabilities were calculated using the equation of Chakraborty et al. (1974) to compute the probability of exclusion based upon at least two diagnostic markers. The exclusion probability (the probability that any one randomly chosen male can be excluded as the father of a chosen individual) is the most common measure of the potential of a given genetic system for use in paternity analysis (Lewis and Snow 1992). An exclusion diagnostic marker (also termed diagnostic peak or diagnostic fragment) is defined by the situation in which, for a given locus, both a potential father and the true mother lack the allele (fragment absent in the AFLP profile) while the particular offspring has it (fragment present in the AFLP profile). Following Pena and Chakraborty (1994), the situation in which a male can be unambiguously excluded as the father is when two or more than two exclusion diagnostic markers are revealed when analyzing the AFLP profiles of the potential father, the true mother, and the offspring, thus allowing for one mutation in the AFLP profile of the true sire without exclusion.

We calculated exclusion probabilities at the adult population level for highly polymorphic loci ($q^2 > 3/N$). For k markers, the cumulative probability Q of exclusion for at least one diagnostic marker (i.e., the probability of exclusion based on the criterion of the detection of only one diagnostic marker to exclude a nonfather), is:

$$Q = 1 - \prod_{i=1}^{k} (1 - \pi_i). \qquad (2)$$

The exclusion probability P on at least two diagnostic markers (i.e., the probability that a randomly chosen male can be excluded as the father of a chosen offspring under the cri-

terion of the detection of two or more diagnostic markers in the comparison of the profiles among the offspring, the mother, and the potential father), is:

$$P = Q - \sum_{i=1}^{k} \pi_i \prod_{\substack{j=1 \\ j \neq 1}}^{k} (1 - \pi_i), \quad (3)$$

with π_i being the probability of exclusion based upon the ith marker; that is, the probability of having no peak in both parents ($q_i^2 \times q_i^2$) and one allele present p_i in a nymph:

$$\pi_i = q_i^2 q_i^2 p_i. \quad (4)$$

Determination of paternity, distribution of diagnostic fragments, and verification of the paternity assignment process.– The procedure we used to determine paternity was based on the determination of exclusion diagnostic markers (diagnostic peaks in the AFLP profile), where a peak (fragment) was absent in both parents (i.e., when both are recessive homozygotes) and present in the nymph. The male (in this case the last male to copulate with a female) was excluded as the genetic father of the nymph when at least two diagnostic peaks were found in the nymph-mother–potential father profile. We followed Westneat (1990, 1993) and Chuang et al. (1999) to estimate mutation rates and to check out the criterion of using a threshold of at least two diagnostic fragments to exclude a male as the father of a nymph.

For the study of relatedness among individuals we did not use the similarity index (Lynch 1990; Lynch and Milligan 1994) but we calculated a pairwise genetic dissimilarity matrix for all individuals from the AFLP data by Euclidean distance (Krauss 1999) using NTSYSpc 2.02i software (Exeter Software, New York). Euclidean distance between individuals i and j for all loci x (where x_{ki} and x_{kj} are equal to either one or zero) is:

$$E_{ij} = \sqrt{\sum_{k=1}^{n} (x_{ki} - x_{kj})^2}. \quad (5)$$

We verified the procedure of paternity assignment for those nymphs for which we concluded that they were sired by the last male to copulate with the female. The correct assignment relies on a higher relatedness between the nymph and the nonexcluded father than expected by chance. We compared the genetic dissimilarity (estimated from Euclidean distance) across four levels of parentage: (A) nymph-assigned father; (B) nymph-mother; (C) nymph-males others than the assigned father (all males are individuals with probability $p > 0$ of being the nymph's father because the nymph's mother could have mated with any of them in the field); and (D) nymph-females others than the mother (these females have probability $p = 0$ of being the nymph's mother). In these comparisons the data are not independent because for a given nymph there was only one value of relatedness with the father or with the mother and a series of values for the relatedness with other males or females (Danforth and Freeman-Gallant 1996). Thus, it was necessary to control by nymph when analyzing differences in genetic relatedness. We carried out a mixed model of variance analysis (Statsoft 1996), in which nymph was entered as a random factor and parentage as fixed factor with the levels A to D. This analysis was preferred over a *t*-test for dependent samples because the latter uses the mean genetic value for each group of values for a nymph and does not consider variance of data in levels C and D.

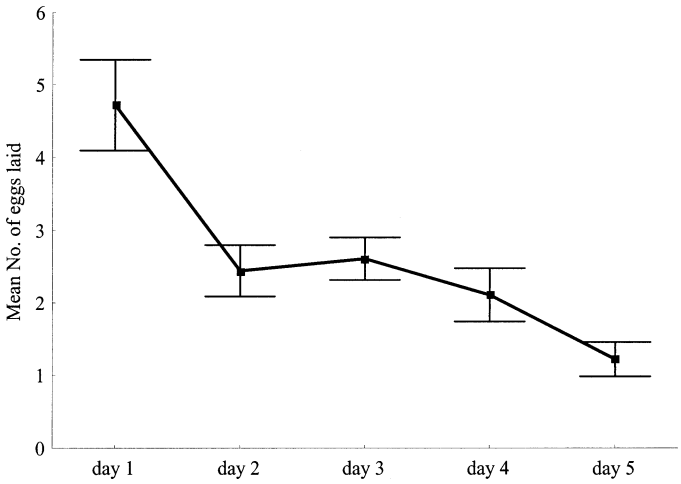

FIG. 1. Mean oviposition rate of females (±SE, $n = 18$) along the experimental period.

The number of eggs laid and the proportion of eggs fertilized by the last male (P_n) were used as dependent variables in repeated measures ANOVAs. These variables were log-transformed and arcsine-transformed, respectively, to fulfill parametric assumptions (Sokal and Rohlf 1981). Other assumptions of the repeated measures ANOVA were checked by specific tests; the compound symmetry assumption was checked using the tests of Greenhouse-Geisser and Huynh-Feldt, and the sphericity assumption was checked by Mauchley's sphericity test (Statsoft 1996).

RESULTS

Oviposition after Copulation

The females from the 18 pairs laid a total of 236 eggs throughout the 5-day period. The mean number of eggs laid by females during this period was 13.1 eggs (SE = 0.7, minimum = 9, maximum = 21). The number of eggs laid decreases as the time from the end of the last copulation increases (repeated measures ANOVA, $F_{4,68} = 7.82$, $P \ll 0.001$; Fig. 1). The first day after copulation females laid 36.02% of the eggs laid throughout the laying period monitored.

Despite the extremely short time interval between the end of copulation and experimental male removal, 41.2% (35 of 85) of the eggs laid by females in the first day after copulation were laid on mating males. A total of 227 of the 236 eggs laid hatched, with an average proportion of eggs hatched of 96.1% (SE = 1.9, minimum = 69.2, maximum = 100, $n = 18$).

Paternity of Eggs and Sperm Precedence Patterns

Within the study sample (109 individuals) two AFLP primer pairs generated a total of 116 scorable fragments (see Table 1). The mean number of fragments generated per individual was 52 (SE = 0.9, $n = 109$). All scored fragments were

TABLE 1. Polymorphism for two amplified fragment length polymorphism primer pairs in a sample of 109 individuals (10 males, 10 females, 89 nymphs) of *Phyllomorpha laciniata* from central Spain, with mean dominant allele frequencies (p) and standard error (SE) calculated for adults (20 individuals) for polymorphic loci with $q^2 > 3/N$ (for details see text).

Primer pair	No. of fragments	Polymorphic fragments	Mean (p)	SE
MseI-CAT/EcoRI-AAG	56	56	0.275	0.02
MseI-CAG/EcoRI-AAC	60	60	0.204	0.02
Total	116	116	0.239	0.02

polymorphic, as assessed by band absence in at least one individual. We used 91 highly polymorphic loci (with $q^2 > 3/N$, with N being the number of adult individuals) to calculate allele frequencies. Dominant allele frequencies (p), calculated from the frequency of the recessive phenotype q^2 (band absence) for each one of these loci, varied from 0.0 (band absence in all adults) to 0.55 (band absence in four of the 20 adults). The mean dominant allele frequency (p) over 91 polymorphic loci generated from 20 adults was 0.239 (see Table 1). The global exclusion probability based on at least two diagnostic peaks (P), calculated for the combination of the two primer pairs, MseI-CAT/EcoRI-AAG and MseI-CAG/EcoRI-AAC, was 0.98. The global exclusion probability considering a single diagnostic peak to exclude paternity by the last male to mate with a female (Q) was 0.997.

To determine the proportion of eggs fertilized by the last male (P_n), 83 nymphs, distributed among seven families, were analyzed. The proportion of eggs analyzed out of the total number of eggs laid by the females of these families was 87.4%. We resolved AFLP profiles for these nymphs and for the females and males of these families. The 83 nymphs analyzed possessed between zero and 12 fragments present in the nymph and not present in both the mother and the last mating male (i.e., diagnostic fragments; see Fig. 2). In 33 nymphs of 83 there was none or a single diagnostic fragment.

We estimated mutation rates and checked out the criterion of using a threshold of at least two diagnostic fragments to exclude a male as the father of a nymph. This estimation was difficult in this case, because the distribution was continuous and not bimodal, as can be seen in Figure 2 (see also Westneat 1993). We conservatively assumed that in the case of nymphs with only one diagnostic fragment the father was unlikely to have been assigned erroneously. Fifty-nine of the 83 offspring had one or more diagnostic peaks when compared with the mother and the last male to copulate with the mother. Using observed proportions of zero and one diagnostic fragments and assuming that all cases of a single novel band represent mutations, we calculated the average mutation rate (m) to be 0.27 per individual per meiotic event. The mutation rate per locus (μ), calculated by dividing m by the average number of fragments scored per lane (52), was 0.005 per band per meiotic event. The expected probability of observing two diagnostic fragments from mutation alone is $0.27^2 = 0.07$ and three diagnostic fragments is $0.27^3 = 0.02$. Given the number of nymphs analyzed, we thus expected about six and two nymphs to have two and three diagnostic fragments, respectively. The observed values for two and three diagnostic fragments were greater than expected (see Fig. 2). Thus, we conclude that nymphs with two or more diagnostic fragments were highly likely to be sired by another male than the last male to copulate with the female.

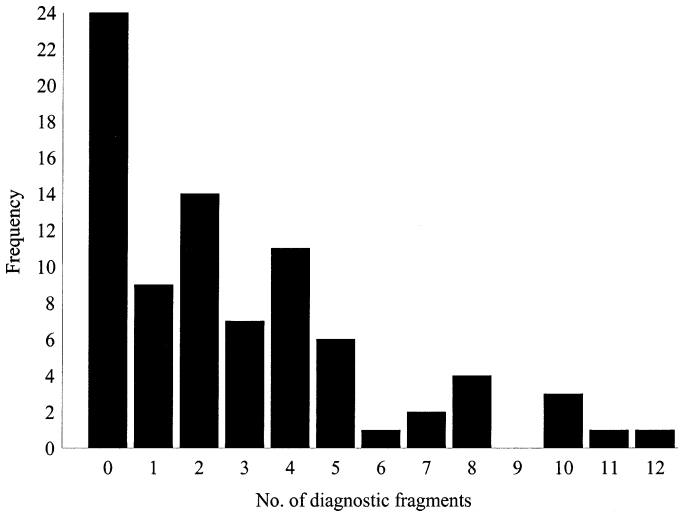

FIG. 2. Distribution of the diagnostic fragments between the profiles of the nymph-mother-last mating male, for the families analyzed to determine P_n.

We verified the procedure of paternity determination comparing the genetic dissimilarity across the levels of parentage A (nymph-assigned father), B (nymph-mother), C (nymph-males others than the assigned father), and D (nymph-females others than the mother). There were highly significant differences among these levels (mixed model ANOVA, $F_{3,96} = 57.7$; $P \ll 0.000001$). As expected, genetic similarity between nymph-assigned father (A) was not different than for nymph-mother (B), whereas both were different than that for groups C and D (see Fig. 3). Thus, based on the level of acceptance defined in the Materials and Methods section, and on the probability of exclusion obtained, 39.8% of the nymphs were considered as sired by the last mating male.

The average proportion of eggs fertilized by the last mating male for each family over the whole laying period varied from 0.1 to 0.78% (see Table 2). The mean P_n obtained from the values for each family was 0.43 (SE = 0.11); thus, this value represents the confidence of paternity for the whole sample. The proportion of eggs sired by the last male calculated for each one of the five days varied from 0.0 to 1.0 (see Table 2). An analysis of the variation of P_n shows that there are no differences in the proportion of eggs sired by the last male (arcsine-transformed) between days (repeated measures ANOVA, $F_{4,16} = 0.13$, $P = 0.97$). The proportion of variance in the dependent variable that is explained by differences among groups is 0.031 (as assessed by the eta-squared coefficient), that is, a variance of 3% of P_n would be explained by the effect time after the last copulation in case of statistical significance. The repeated measures analysis did not include families 5 and 6 because in family 5 the only egg laid on day 5 could not be analyzed, and in family 6 no eggs were laid on day 2 (see Table 2). This implies empty cells, thus repeated measures cannot be conducted for these families. Calculating the mean P_n of all families (in-

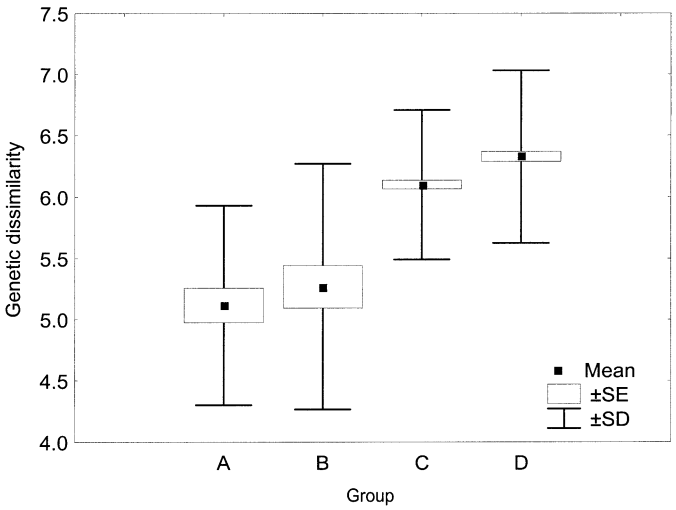

FIG. 3. Genetic dissimilarity for the relationships between A (nymph-assigned father, for 33 nymphs sired by the last male to mate with the female, 33 pairwise comparisons), B (nymph-mother, for 33 nymphs sired by the last male to mate with the female, 33 pairwise comparisons), C (nymph-males others than the assigned father; 33 nymphs, 297 pairwise comparisons), and D (nymph-females others than the mother; 33 nymphs, 297 pairwise comparisons). *P*-values (mixed model ANOVA, unequal N HSD post hoc test) for the comparison of the genetic dissimilarity, as estimated from Euclidean distance, across these groups are the following: A–B, $P = 0.75$; A–C, $P \ll 0.0001$; A–D, $P \ll 0.0001$; B–C, $P \ll 0.0001$; B–D, $P \ll 0.0001$.

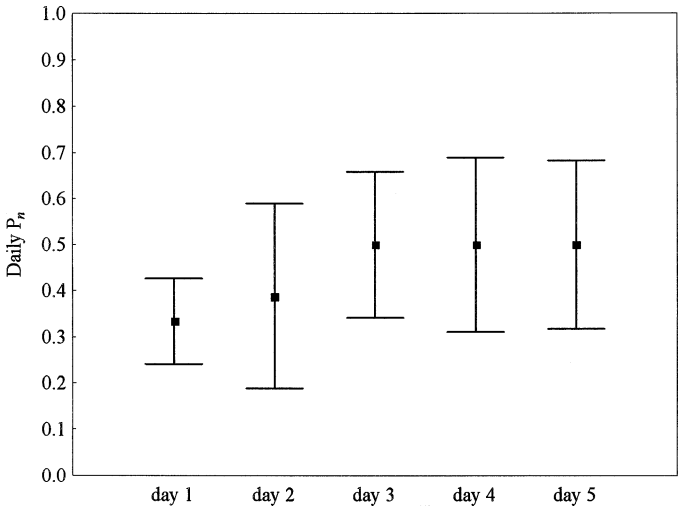

FIG. 4. Mean proportion (±SE) of eggs sired by the last mating male (P_n) calculated from the daily P_n of the families analyzed for the determination of the sperm precedence patterns.

cluding families 5 and 6) for each day clearly supports that P_n remains nearly constant as the time after the last copulation increases, for the laying period considered (see Fig. 4).

Paternity of Eggs Carried by Males

Eggs laid on males during the short interval between the end of copulation and their experimental removal were processed. In addition to the eggs carried by males in those families included in the study of sperm precedence patterns, eggs on males from another two families were included. The same procedure of determination of paternity based on the number of diagnostic peaks that has been described above was conducted for this set of data. A percentage of 93.33% of the eggs carried by males in these families were analyzed. Among the eggs carried by males, a mean percentage of 38% (SE = 12.16) were their own offspring.

DISCUSSION

Mechanisms of Sperm Utilization

The results of this study show that in naturally occurring *P. laciniata* the last male to mate with a female experiences an intermediate level of fertilization success (mean P_n = 0.43), characterized by a moderate variance (SD = 0.30, range = 0.1–0.78). In addition, our results show that the averaged P_n-value remains constant throughout the 5-days laying period.

Our results strongly suggest that a mechanism of sperm mixing is responsible for the pattern of sperm precedence in the golden egg bug. The patterns of sperm use in studies on sperm competition are typically inferred from the mean P_2-value (Birkhead and Møller 1998; Simmons 2001). Intermediate values are usually taken as indicative of sperm mixing, while extreme values are assumed to be the result of mechanisms such as sperm stratification, sperm removal, or sperm displacement by sperm flushing (Birkhead and Møller 1992; Simmons and Siva-Jothy 1998; Simmons 2001). All these mechanisms result in high P_n-values, which may either remain constant with time (e.g., sperm displacement and sperm removal) or may decrease with time (e.g., sperm stratification or incomplete sperm displacement followed by sperm mixing). Recently, it has been acknowledged that intraspecific variation in the proportion of offspring sired by the last male to copulate with a female provides additional information about the mechanisms involved in sperm use (Lewis and Austad 1990; Cook et al. 1997; Simmons and Siva-Jothy 1998; Harvey and Parker 2000; Simmons 2001). Low variance is expected where there are mechanisms to preempt previously stored sperm through for instance sperm displacement or if there is a depletion of the sperm-storage organ prior to the second mating. On the contrary, moderate variance is expected when sperm mixing occurs, being the magnitude of variation associated with the degree of sperm mixing. Thus, the moderate degree of variance found in the golden egg bug also supports the idea that sperm from the different males who mate with a female mix in the spermatheca, which results in spermatozoa from different males fertilizing the ova in a rather unpredictable way. This, in turn, implies variable patterns of paternity with no order effects. The intermediate P_n-value of *P. laciniata* and its moderate variance are close to those found for other heteropterans such as *Oncopeltus fasciatus* (Economopoulus and Gordon 1972), *Jadera haematoloma* (Carroll 1991), and *Nezara viridula* (McLain 1985).

Because sperm competition mechanisms are largely the result of female reproductive morphology and physiology (Birkhead and Møller 1998), it is females who define the arena in which ejaculates compete, and thus the rules of the

TABLE 2. Number of eggs included in paternity analyses (N), number of eggs fertilized by the last mating male (F), and the proportion of eggs sired by the last mating male (P_n) for the whole 5-day laying period, for each particular family.

Family	Day 1 F/N	Day 2 F/N	Day 3 F/N	Day 4 F/N	Day 5 F/N	Days 1–5 P_n
1	1/8	1/3	0/2	0/1	1/2	0.19
2	0/2	0/3	1/2	0/2	0/1	0.10
3	2/3	0/2	1/3	0/3	0/2	0.23
4	1/7	0/1	0/2	2/2	1/2	0.29
5	2/4	1/1	3/3	1/1	0/0	0.78
6	2/5	0/0	2/3	2/2	2/2	0.67
7	1/2	1/1	3/3	1/2	1/1	0.78
Total	9/31	3/11	10/18	6/13	5/10	0.40

game (Gomendio et al. 1998). Thus, it is worth asking why sperm mixing is the mechanism favored by female genital morphology in a species in which females benefit considerably from male care. It has been widely accepted that when females benefit from male investment in offspring, mechanisms that ensure high levels of confidence of paternity should be favored and such mechanisms tend to be those resulting in high levels of last-male precedence. When females lay one or a few clutches of eggs it may be a good option to ensure high levels of paternity to the last male to copulate and in this way maximize the chances that he will accept the whole clutch after copulation. This is the case in a few well-known examples such as male giant water bugs, who become willing to accept clutches being laid on their backs only after copulation takes place (Smith 1979; Smith 1997). Thus, the fact that sperm mixing occurs in *P. laciniata* and that it results in intermediate levels of paternity seems paradoxical.

The key difference between *P. laciniata* and previous models is that *P. laciniata* females do not lay clutches, but rather lay eggs sequentially over several months (between zero and 10 eggs laid each day). Thus, it is not in their interest to ensure that males will accept eggs at a few specific points in time (e.g., after copulation); on the contrary, they need to maximize the chances that each time an egg is laid there will be a male willing to accept it. In this context it may be a better strategy for females to develop a mechanism that results in sharing of paternity between all the males with whom they have copulated than a mechanism that gives high paternity to just one male. In addition, sperm mixing implies that, as long as other copulations do not take place, paternity remains constant through time, which means that males will tend to accept eggs not just after copulation but also for longer periods. Finally, sperm mixing implies that it is impossible to predict when sperm from a particular male will fertilize an egg. Thus, males are left with no cues about when their own offspring will be produced. This explains why the temporal patterns of egg acceptance by males are so flexible, and why they make no effort to mate guard the female after copulation.

However, because females take advantage of the opportunity of having a willing male around after copulation, egg laying is enhanced at this time, which results in a high proportion of eggs being laid on males some time after they have copulated with a female. Our results show that just after copulation has ended females tend to lay a greater number of eggs: the first day after copulation females laid two to three times more eggs than during the following four days. In our study, 41.18% of eggs laid the first day were laid on males, despite the short interval between the end of copulation and the experimental removal of males. The proportion of these eggs that were sired by the carrying male was 38%.

Paternal Care and Confidence of Paternity in Natural Populations of the Golden Egg Bug

Our results indicate that in natural populations a male who copulates with a female will enjoy an intermediate confidence of paternity that remains constant with the time, that is, it is expected that he would sire on average around 43% of the offspring produced by the female, independently of the time since the last copulation. This raises the question as to why males are willing to carry eggs if confidence of paternity is intermediate. According to the model by Westneat and Sherman (1993) males are predicted to decrease their parental effort as a response to a reduction in paternity under the following conditions.

Males can discriminate their own young and may choose to care only for their true genetic offspring.—In general, data from bird studies suggest that males are not able to discriminate their own from unrelated chicks (see Westneat and Sherman 1993; Kempenaers and Sheldon 1996). Instead, males tend to rely on indirect cues to estimate probability of paternity in the brood as a whole, such as the proportion of exclusive mating access with the female (Burke et al. 1989; Davies et al. 1992). Male golden egg bugs are unlikely to be able to discriminate eggs sired by them from unrelated eggs. More importantly, a sperm mixing mechanism implies that the process by which sperm from different males present in the spermatheca fertilize the eggs is random, and therefore that there are no cues that males can use to predict when their own offspring will be produced. Because paternity does not vary for several days after copulation, there is no particular time when the probability of accepting a true genetic offspring is greater.

Survival costs to offspring as a result of male desertion are not high.—Whittingham et al. (1992) predicted that when male care is critical to offspring survival, males might provide care in an all-or-nothing way, caring for young unless confidence of paternity falls below a very low threshold, and empirical tests have found support for this prediction (Whittingham et al. 1993; MacDougall-Shackleton and Robertson 1998). It is clear now that in *P. laciniata* eggs laid on plants suffer very high mortality rates: only around 3% of the eggs laid on plants survive until hatching due to parasitation and predation, while 25% of eggs laid on adults survive (Reguera and Gomendio 2002). Thus, male caring is crucial for offspring survival.

Males know their own paternity, and confidence of paternity is expected to improve in future breeding attempts.—Westneat and Sherman's (1993) model predicts that, if individuals' levels of paternity in future reproductive episodes are, on average, the same as in the present, there will be nothing to be gained by males reducing their levels of care in any one breeding attempt. In a similar way, Maynard Smith (1978)

and Grafen (1980) pointed out that if paternity is the same in all breeding attempts, then it should have no effect on the optimal form of parental behavior. In the golden egg bug system sperm mixing implies that males have no cues to assess their levels of paternity, so they cannot evaluate how they should adjust their effort. In addition, males can do little to improve their level of paternity in different breeding attempts because when sperm from different males mix in the spermatheca there is no way for males to reach high paternity levels, because all males share paternity for long periods of time. The lack of better paternity prospects in the future, together with the lack of cues to assess paternity, thus explains in part why males accept eggs at intermediate levels of confidence of paternity.

In most cases the main cost for males of investing in offspring is a reduction in the possibilities of mating with other females (Zeh and Smith 1985; Clutton-Brock 1991; Smith 1997), and male care is expected to evolve when such cost is low, either because remating opportunities are rare (Owens 2002) or because male care does not imply a substantial loss of mating opportunities. Male *P. laciniata* do not suffer a reduction in mating opportunities because egg-carrying males continue to search for other females and mate with them. This is because eggs are glued on the backs and males do not have to remain in a particular place looking after the eggs, neither does egg carrying imply any additional activities that could reduce the time invested in mate searching and copulation (Gomendio and Reguera 2001). The reduction in mobility that egg carrying entails has no effect upon mate searching, because it only limits the ability of males to fly, a rare behavior that only occurs when adults have to escape from a predator (Gomendio and Reguera 2001). Thus, egg carrying by males does not entail a cost in terms of mating effort, but it does imply a survival cost to males who become more vulnerable to predators (Reguera and Gomendio 1999; Kaitala et al. 2000).

In summary, female golden egg bugs are polyandrous and store sperm in the spermatheca. When a male copulates with a nonvirgin female, his probability of siring offspring is around 0.43 for at least the following five days if no other copulations take place, with a moderate degree of variance. This strongly suggests that sperm mixing is the most likely sperm competition mechanism. Males are unlikely to distinguish eggs sired by them from unrelated eggs and have no cues to predict when their own eggs will be laid; thus, males are unable to accept their own eggs exclusively. Despite the intermediate levels of confidence of paternity males accept eggs because sperm mixing implies that there are no chances of improving confidence of paternity levels in future breeding attempts and because eggs suffer very high mortality rates when laid on plants. For males the risk of mistakenly leaving one's own offspring on plants seems higher than the risk of accepting unrelated eggs. In addition, males do not suffer a loss of opportunities to mate with other females while they carry eggs, which tends to be the main cost preventing the evolution of male care. The prolonged copulations observed in this species may be the only way in which males may attempt to maximize the number of sperm transferred, the chances that the female will lay eggs soon after copulation, and the likelihood that by accepting them offspring survival will be improved.

Given the low survival prospects that eggs face when laid on plants, it is worth considering briefly why female care has not evolved in this species. Females cannot lay eggs on themselves, but they could lay eggs on plants and look after them. This would imply laying clutches of eggs, rather than one egg at a time. However, the costs to females in terms of a reduction in fecundity would be very high, given their high fecundity and extreme iteroparity. In addition, it is unlikely that an herbivorous insect would have the ability to defend its offspring against a parasitoid wasp, the main cause of egg mortality on plants and the one that is alleviated when eggs are carried by conspecifics. Thus, in this species the costs to females of producing discrete clutches and reducing their fecundity are probably much greater than the costs to males of carrying eggs, given that their chances of mating with other females seem unaffected. This sex asymmetry in the costs of caring for eggs has probably favored the evolution of male egg carrying in this species, despite the intermediate levels of paternity enjoyed by males.

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